Prevention of Influenza Pneumonitis by Sialic Acid–Conjugated Dendritic Polymers

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Influenza A viral infection begins by hemagglutinin glycoproteins on the viral envelope binding to cell membrane sialic acid (SA). Free SA monomers cannot block hemagglutinin adhesion in vivo because of toxicity. Polyvalent, generation 4 (G4) SA-conjugated polyamidoamine (PAMAM) dendrimer (G4-SA) was evaluated as a means of preventing adhesion of 3 influenza A subtypes (H1N1, H2N2, and H3N2). In hemagglutination-inhibition assays, G4-SA was found to inhibit all H3N2 and 3 of 5 H1N1 influenza subtype strains at concentrations 32–170 times lower than those of SA monomers. In contrast, G4-SA had no ability to inhibit hemagglutination with H2N2 subtypes or 2 of 5 H1N1 subtype strains. In vivo experiments showed that G4-SA completely prevented infection by a H3N2 subtype in a murine influenza pneumonitis model but was not effective in preventing pneumonitis caused by an H2N2 subtype. Polyvalent binding inhibitors have potential as antiviral therapeutics, but issues related to strain specificity must be resolved.

Most viruses use a receptor that binds to a cellular surface component as a targeting mechanism. Theoretically, blocking the initial interaction of a virus with this host cell receptor can prevent viral infection. This binding may be inhibited by an extracellular therapeutic agent that resembles the surface-binding component of the host. Influenza A virus is an enveloped virus with a segmented, single-stranded, RNA genome. Annual epidemics and occasional pandemics of influenza A virus are a significant health concern [1]. These viruses have evolved some exceptionally effective survival mechanisms and are able to circumvent the immune response because of the highly mutable genes that encode their surface proteins. Segment 4 of the influenza A genome encodes the major surface glycoprotein, which is called hemagglutinin because of its ability to agglutinate erythrocytes [2]. Hemagglutinin consists of 2 structural domains, with the peripheral, globular domain having a binding cleft for attachment to cells [3]. Antigenic drift mutations and antigenic shift of the gene segments that encode hemagglutinin and neuraminidase contribute to the preservation of this virus and present problems for vaccine and small-molecule therapeutic development [1, 4]. Sialic acid (SA) molecules present on cellular surface structures (glycoproteins or glycolipids) are the targets for binding by hemagglutinin (figure 1). This binding action is the crucial component for the initiation of infection and, therefore, serves as a potential target to decoy therapy with effectiveness across different subtypes.

A decoy approach to prevent influenza infection might use monomeric SAs or methyl sialosides to block viral adhesion to cells. However, these monomers become susceptible to rapid enzymatic breakdown in vivo and do not effectively compete with polymeric sialosides on host cells. Thus, high concentrations of monomeric SA must be delivered to inhibit viral infection, and these concentrations are toxic [4–6]. A potential solution could involve conjugating SA molecules to a macromolecule to increase delivery of SA while reducing cytotoxicity. Polyamidoamine (PAMAM) dendrimers are monodispersed, water soluble, macromolecules that are highly branched and well defined [7]. They posses multiple-surface functional groups that can provide an attachment site for SA. Previous experimental data from our laboratory suggest that PAMAM dendrimers [8] are a useful scaffold that can be coupled to make polymeric SA conjugates that will inhibit influenza hemagglutinin protein binding at reduced concentrations [4]. The polymeric interaction of many SA molecules with many hemagglutinin proteins on the virus increases the avidity of these materials for the virus and thus reduces the concentrations of SA subunits needed to prevent viral adhesion in vitro.

As an extension of our previous studies, we evaluated the ability of generation 4 (G4) PAMAM dendrimers conjugated

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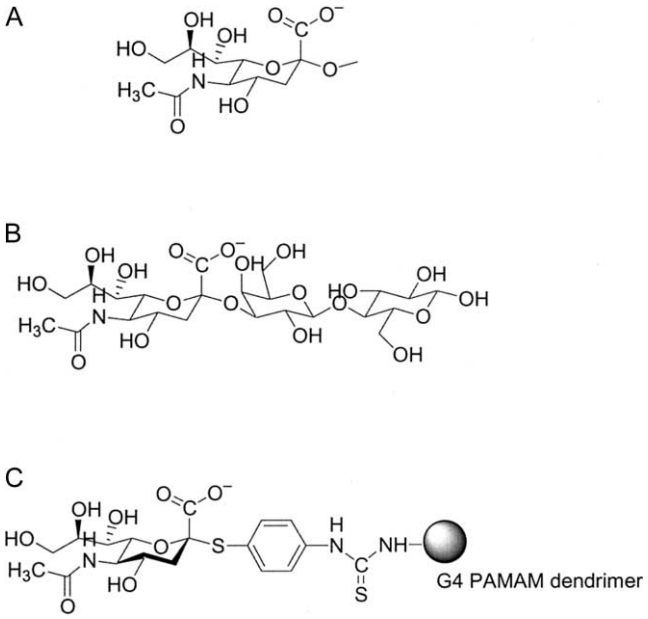

Figure 1. *A,* Sialic acid (SA) unit. *B,* 3′-sialyllactose. *C,* Generation 4 (G4) SA-conjugated polyamidoamine (PAMAM) dendrimer.

with SA (G4-SA; figure 1) to prevent viral adhesion and infection. Three separate approaches were employed. First, we screened the efficacy of the polymeric SA decoy against various serotypes of all 3 major human influenza subtypes—H1N1, H2N2, and H3N3—in vitro by use of a hemagglutination-inhibition assay (HIA). Second, we established a murine model to investigate the ability of dendrimer conjugated with SA to prevent experimental influenza A pneumonitis in vivo. Finally, we calculated molecular models of dendrimers and their conjugates, to ascertain the specific interactions of these molecules with hemagglutinin. The results of these studies suggest that there are great variations in the ability of different hemagglutinins to interact with the decoy molecules.

Materials and Methods

Virus. Influenza A X-31 (A/Aichi/2/68 x A/PR/8/34) H3N2 virus was kindly provided by Alan R. Douglas (National Institute for Medical Research, London). The X-31 virus was adapted for mice through 8 passages in our laboratory, to increase the infectivity in mice (data not shown), according to standard protocols [9]. Increasing the virulence lowers the concentration needed to infect mice and better represents a virulent pathogen. Human virulent influenza A/AA/6/60 (H2N2) and influenza A/AA6/60 (H2N2) mouse-adapted viruses were kindly provided by Hunein Masssab (Department of Public Health, University of Michigan, Ann Arbor). Both viruses were then propagated in embryonated chicken eggs, according to methods described elsewhere [9]. Infectious allantoic fluid was collected and centrifuged at 800–1800 *g* for 10 min. The supernatant was screened for bacterial contamination by culture on blood agar plates, pooled, assessed for titer of hemagglutination units (HAU)/plaque-forming units, and stored at −80°C until used.

The following influenza A subtypes and strains were purchased from American Type Culture Collection: subtype H1N1, strains **PR/8/34**, **Weiss/43**, *FM/1/47, NWS/33,* and *WS/33;* subtype H2N2, strain **A2/Japan/305/57**; and subtype H3N2, strains **Hk/8/68** and **Aichi/2/68**. Viruses shown in boldface type indicate those strains that were propagated in embryonated chicken eggs using methods described above. Viruses shown in italic were used directly from stock cultures.

Dendrimer synthesis, conjugates, and controls. PAMAM dendrimer synthesis has been fully described elsewhere [4, 7]. G4 PAMAM dendrimers were assembled. Initially, G0 was made by adding methyl acrylate to ethylenediamine (EDA) via Michael addition, followed by amidation of the tetraester product with an excess of EDA. To produce the G4 product, this process of alternating Michael addition with amidation reactions was repeated 4 more times. The final molecular weight was 14,215 Da, with a diameter of 45 Å. Dendrimer characterization was accomplished by use of ^{1}H and ^{13}C

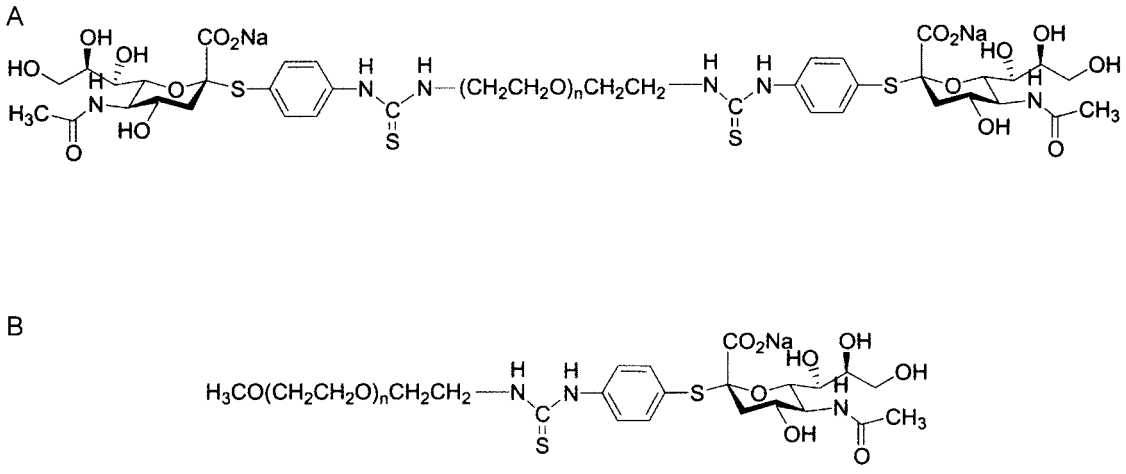

Figure 2. *A,* Bis-amino polyethylene glycol (PEG) with 2 sialic acid (SA) conjugates. *B,* Methoxy/amino PEG with SA conjugate.

Table 1. Murine dose-dependent survival response with generation 4 sialic acid–conjugated polyamidoamine dendrimer (G4-SA) and influenza A X-31 (H3N2) mixtures.

Virus strain (subtype), treatment	Virus dose		Mortality, no. of mice that died/ total no. (%)	Duration of survival, mean days
	log pfu	pfu/mouse		
AA/6/60 (H2N2)				
Virus alone	3.7	5×10^3	5/5 (100)	6.4
Virus and G4-SA	3.7	5×10^3	6/6 (100)	6.8
Virus alone	4.7	5×10^4	12/13 (92)	5.7
Virus and G4-SA	4.7	5×10^4	11/13 (85)	6.5
Virus alone	5.4	2.5×10^5	5/5 (100)	4.0
Virus and G4-SA	5.4	2.5×10^5	5/5 (100)	4.0
X-31 (H3N2)				
Virus alone	6.0	1×10^6	16/17 (94)	5.0
Virus and G4-SA	6.0	1×10^6	0/17 (0)	⩾14.0
X-31 (H3N2)				
Virus alone	2.0	100	5/5 (100)	5.8
Virus and G4-SA	2.0	100	1/5 (20)	⩾13.0
Virus alone	1.4	25	10/10 (100)	7.0
Virus and G4-SA	1.4	25	1/10 (10)	⩾13.4

NOTE. Every mouse that was given G4-SA received 9 μg/g of body weight.

nuclear magnetic resonance (NMR) spectroscopy, size exclusion chromatography–refractive index, matrix-assisted laser desportion/ionization–time-of-flight mass spectroscopy, and multi-angle laser light scattering. Dendrimer conjugates were made using SA, Tris, and ethanol, by a deprotection of the blocking groups and then by conjugation via an aromatic thiourea linker [4]. The final, fully-conjugated dendrimer G4-SA had a molecular weight of 43,495 Da, and the conjugates were characterized by UV-visible and ^{1}H and ^{13}C NMR spectroscopy. Tris- and ethanol-conjugated dendrimers were used as negative controls. The dendrimers were reconstituted with PBS (LifeTechnologies, GibcoBRL) and mixed overnight on a tube rotator at room temperature. Further storage was at −70°C.

Molecules known to inhibit influenza A adhesion were purchased from Sigma for use as positive controls (SA, and 2-*O*-methyl sialoside); 3′-*N*-sialyllactose (Calbiochem) was also assayed because of its potential use as a conjugate (figure 1). Two linear, polyethylene glycol (PEG) linker chains (Shearwater Polymers) were used with SA attached, to test a conjugate with the SA extended from the surface of the polymer (figure 2).

HIA. Assays followed procedures described elsewhere [9, 10]. Chicken erythrocytes at a 0.5% concentration in PBS were used for hemagglutination detection. Flexible, round-bottom 96-well plates were used for serial dilutions, with PBS used as a control. Virus titer was established at 4 HAU/25 μL of diluted virus stock by back-titration before every assay, using SA and sialoside as positive controls. Virus dose was fixed as 4 HAU across the entire assay. As additional controls, each test substance was assayed without virus, and the virus was assayed without any test substance. All substances were assayed in a 25-μL volume. All controls were added at a concentration of 40 m*M,* resulting in an initial concentration of 10 m*M* in the first well after dilution; 3′-*N*-sialyllactose was also assayed as an inhibitor. G4-SA was initially added at a concentration of 2.5 m*M* and was serially diluted. Virus was incubated with the treatment at 37°C and then assessed for hemagglutination. The end point of the HIA was recorded, and an average of each individual assay was calculated. The end point of the HIA is the concentration at which the virus agglutinates 50% of the red blood cells. Each experiment was internally replicated. To establish consistency, all assays were performed at least 3 times by a masked reader.

Toxicity testing in vivo. To establish the in vivo toxicity range, 24 5-week-old CD-1 mice (Charles River) were divided into 6 groups of 4 mice each. Animal housing was provided with standard water and food in an approved animal facility for at least 2 days before the experiment. Control mice each received 50 μL of PBS administered once intranasally, with slight isoflurane anesthesia, in a bell jar. The volume delivered per nare was 25 μL. The remaining groups were treated with G4-SA in PBS in serial 10-fold dilutions in the same manner, with doses ranging from 0.34 μg/mouse to 0.000034 μg/mouse. The mice were observed for clinical signs of toxicity, including ruffled fur, hunched back, weakness, hard breathing, tachypnea, and cyanosis, for a total of 6 days after inoculation. At day 6, all mice were killed, and their lungs were harvested and fixed for pathologic evaluation.

In vivo virus dose determination. Lethal doses of influenza A viruses, influenza A X-31, and mouse-adapted influenza A X-31 were determined in a mouse pneumonitis model before the dendrimer experiments. LD_{50} testing was performed according to a standard protocol, as described elsewhere [11–13]. In brief, 4–5 week-old specific pathogen–free CD-1 mice (Charles River) of either sex were anesthetized with isoflurane in a bell jar and were administered 50 μL (25 μL/nare) once at serial dilutions of influenza A virus intranasally. Mice were observed for a period of 14 days, with rectal core body temperatures recorded daily by use of a BAT-12 digital thermometer fitted with a RET-3 type T mouse rectal probe (Physitemp). For the LD_{50} determination, the number of mice in each experimental group that survived and the number that died from the infection were recorded at day 14 after virus administration. The LD_{50} values were calculated according to the Reed and Muench method, as described elsewhere [14].

After nasal inoculation with a virulent influenza A virus, mice developed, over the course of 2–3 days, appreciable clinical signs of pneumonia, including piloerection, loss in body weight, hunched appearance, and grouping together. As the infection progressed, these signs became more pronounced, and the mice became unresponsive. When the body temperature of the infected mice decreased to ⩽32°C, mice were killed. The LD_{100} value for mouse-adapted X-31 was found to be 25 pfu/mouse, whereas that for non–mouse-adapted X-31 was found to be 25000 pfu/mouse.

Mouse-adapted influenza A/AA/6/60 virus demonstrated 80%–

Table 2. Effect of generation 4 sialic acid–conjugated polyamidoamine dendrimer (G4-SA) on mice simultaneously infected with 3 strains of influenza A virus of 2 subtypes.

G4-SA, mg/g of mouse body weight	Mortality, no. of mice that died/ total no. (%)	Duration of survival, mean days	No. of mice with plaque-forming virus/total no. (%)
9.0	0/5 (0)	>14.0	0/5 (0)
3.6	0/5 (0)	>14.0	0/5 (0)
1.8	0/5 (0)	>14.0	0/5 (0)
0.9	4/6 (67)	8.3	4/6 (67)
0.18	5/5 (100)	7.0	5/5 (100)
0.018	5/5 (100)	6.2	5/5 (100)
0.0	10/10 (100)	6.6	10/10 (100)

NOTE. Every mouse was given 2×10^4 pfu of virus.

Table 3. Comparison of equivalent sialic acid (SA), concentrations of monomeric SA, sialoside, and generation 4 SA-conjugated polyamidoamine dendrimer (G4-SA) required for inhibition of hemagglutination.

		Positive controls		Negative controls	
Virus subtype	G4-SA	SA	Sialoside	Dendrimer with Tris	Dendrimer with ethanol
H1N1					
PR 8/34	NI	10	10	NI	NI
A/Weiss/43	0.234	10	10	NI	NI
A1/FM/1/47	NI	10	10	NI	NI
A/NWS/33	0.313	10	10	NI	NI
A/WS/33	0.313	10	10	NI	NI
H2N2					
AA 6/60	NI	10	10	NI	NI
AA 6/60 mouse	NI	10	10	NI	NI
A2/Japan/305/57	NI	10	10	NI	NI
H3N2					
X-31	0.125	10	10	NI	NI
X-31 mouse	0.195	10	10	NI	NI
HK 8/68	0.0585	10	10	NI	NI
A/Aichi/2/68	0.117	10	10	NI	NI

NOTE. Data are the minimum concentration (m*M*) of SA equivalents required for inhibition of hemagglutination. The maximum G4-SA concentration is 2.5 m*M* of SA equivalents. Results represent an average concentration of at least 3 trials, and each trial was internally duplicated. The observed maximum range was ±1 well in the 96-well plate, which is equal to a 4-fold difference above or below the reported average value. Negative controls of G4 conjugated with either Tris or ethanol did not inhibit viral hemagglutination. Soluble SA as a positive control always inhibited hemagglutination; however, these concentrations were many times higher than that of G4-SA. NI, not inhibited (i.e., ≤2.5 m*M*).

100% lethality at 5000 pfu/mouse. We used this virus, characterized in previous experiments, from a stock solution [11].

Assessing dendrimer SA conjugates in vivo. Initial tests used 4–5-week-old specific pathogen–free CD-1 mice (Charles River) of either sex. These mice were anesthetized as described above and intranasally administered once either AA/6/60 or X-31 influenza A virus (a lethal dose). The virus was premixed with an equal volume of G4-SA at a dose of 9 μg/g of mouse body weight; 50 μL (25 μL/nare) was the volume used for all tests. As a control, the same influenza A virus suspension was mixed with an equal volume of PBS. The development of viral pneumonia was monitored as described above for 14 days. Mice with core body temperatures that decreased to ≤32°C were judged to be terminally moribund and were killed. When death occurred, a necropsy and a gross pathologic examination were done. The right lung lobes were aseptically removed, weighed, and frozen at −70°C. The left lung lobes were preserved in 10% formalin (Fisher Scientific) for histologic evaluation, embedded in paraffin, sectioned serially into 3-mm slides, and stained by the hematoxylin-eosin method. The results of these tests are presented in table 1.

The dose-response study used 41 mice, divided into 5 groups of 5 mice each, 1 group of 6 mice each, and 1 control group of 10 mice. In this experiment, the X-31 influenza A viruses (suspended in PBS) were mixed with an equal volume of G4-SA (set at 25 μL/nare), to a final concentration that was serially diluted, and were administered once. The first group received a G4-SA concentration of 9 μg/g of mouse body weight, and the other groups received 3.6 μg/g, 1.8 μg/g, 0.9 μg/g, 0.18 μg/g, and 0.018 μg/g, respectively. The seventh group (10 mice) received 2×10^4 pfu/mouse of influenza A X-31 virus alone as a control. All virus concentrations were administered at 2×10^4 pfu/mouse. To determine whether influenza virus was present in mouse lung tissue, we used a modification of a plaque assay described elsewhere [11]. The right lung was homogenized in a 7-mL sterile tissue grinder in serum-free MEM to make a 10.0% (wt/vol) solution. The sample was centrifuged at 800–1800 *g* for 10 min. The supernatant was serially diluted and plated on MDCK cells for plaque assay titration, and virus presence was recorded. The results of these tests are presented in table 2.

Two additional experiments were performed on groups of 5 CD-1 mice dosed with either 50 μL of PBS or 0.34 μg of G4-SA in 50 μL of PBS. The first experiment administered 2×10^4 pfu/mouse of non–mouse-adapted X-31 virus to 2 groups of 5 mice each that had been administered the SA dendrimer intranasally 60–90 min earlier. The second experiment administered 1×10^2 pfu/mouse of mouse-adapted X-31 virus to 10 mice similarly pretreated 90–120 min earlier. As in the other studies, these experiments were ter-

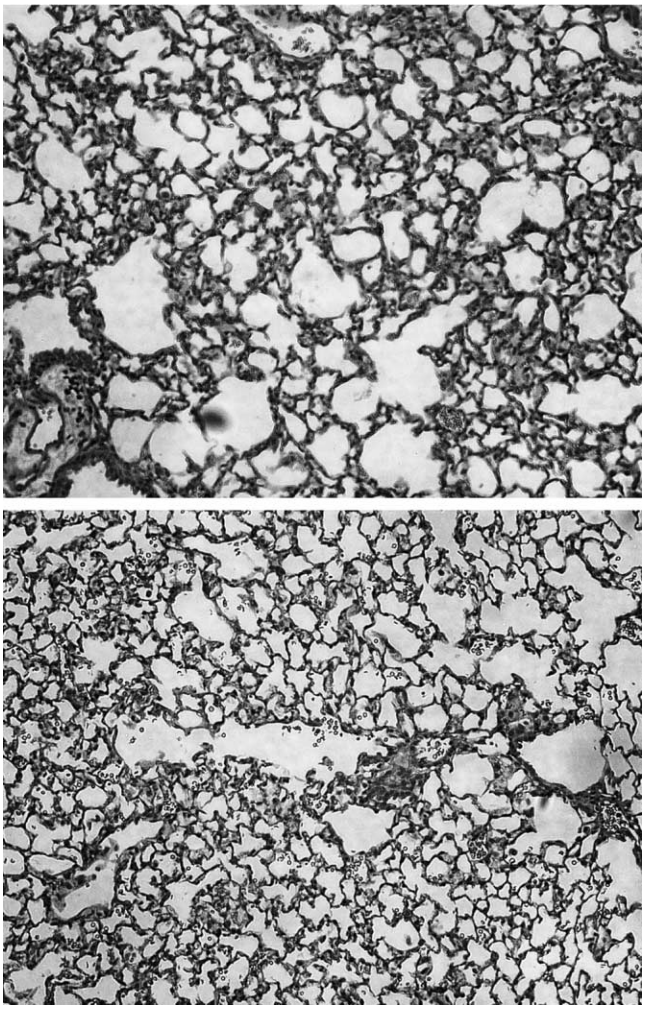

Figure 3. *A,* Mouse lung tissue section 6 days after application of generation 4 sialic acid–conjugated polyamidoamine dendrimer (original magnification, ×200). *B,* Mouse lung tissue section 6 days after PBS control application (original magnification, ×200).

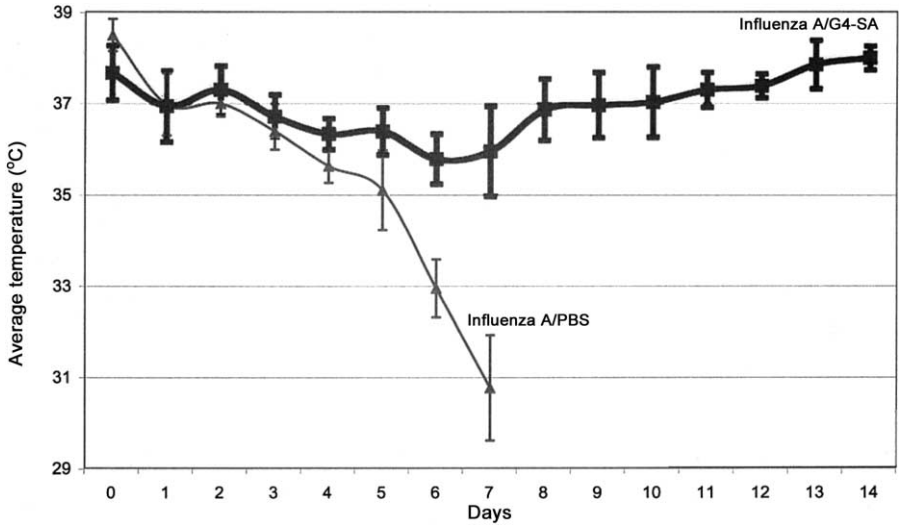

Figure 4. Mouse core temperatures after nasal inoculation with influenza A X-31 or mixed with generation 4 sialic acid–conjugated polyamidoamine dendrimer (G4-SA).

minated either after 14 days or when mice developed core body temperatures ≤32°C and were judged to be terminally moribund.

Computational modeling and simulation of the 3-dimensional structure of hemagglutinin. Hemagglutinin strains A1/FM/1/47 and A/WS/33 (both subtype H1N1), AA 6/60 (subtype H2N2), and A/Aichi/2/68 (subtype H3N2) were analyzed to determine their 3-dimensional structures. We obtained the A/Aichi/2/68 amino acid structure from the Protein Data Bank (available at http://www.rcsb.org/pdb/). The sequences of the other stains were all obtained from the NCBI databank (http://www.ncbi.nlm.nih.gov/). Homology sequences of each strain were searched for in the Protein Data Bank using the BLAST search algorithm (available at http://www.ncbi.nlm.nih.gov/BLAST/). We used the high score structures for references to construct 3-dimensional structures from the proteins amino acid sequence. All models were built on an Onyx workstation (Silicon Graphics) using the Homology module of the Insight II software (Accelrys) and are to be regarded as a computational design.

G4-SA. The molecular model of the G4 PAMAM dendrimer (EDA core) was built on an Onyx workstation using the Insight II software package. All primary amines of the dendrimer were protonated assuming a neutral pH. A molecular dynamics simulation of the model was performed for 100 picoseconds using a consistent valence forcefield (CVFF). SAs were computationally attached to all primary amines of the final configuration of the simulated dendrimer, and a molecular dynamics simulation was performed for 100 picoseconds.

Interaction energy between hemagglutinin and SA-conjugated dendrimer. The primary receptor sites of hemagglutinin and the final configuration of the SA-conjugated dendrimer after the simulation were used for a computation of the interaction energy between hemagglutinin and SA. An SA in the dendrimer conjugate model was manually docked to the primary receptor site of each of the hemagglutinin models. We defined the interacting sites of a hemagglutinin and an SA-conjugated dendrimer within 12 Å from the SA/hemagglutinin interface. The intermolecular energies (including Van der Waals and Coulomb energy) of these interaction sites (total range, 24 Å) were then calculated. The total interaction energy ($E_{\text{interaction}}$) of these interaction sites (within the 24-Å range) was calculated according to the following equation:

$$E_{\text{interaction}} = \sum_i \sum_j \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} + \frac{q_i q_j}{\varepsilon r_{ij}} \right) ,$$

where i is an atom in the hemagglutinin, j is an atom in the SA-conjugated dendrimer, r_{ij} is the distance between i and j, q is the partial charge on the atom, ε is the dielectric constant, and A and B are the van der Waals energy parameters of CVFF [15].

Data analysis. All HIAs were performed at least 3 times. Significance for HIA is represented by a ≥4-fold change in titer. Averages for these titers were determined and reported. The 50% effective dose was calculated using a logistic regression model, with a confidence interval of 0.61–1.51 (table 2).

Results

HIA. Hemagglutination with influenza A viruses of subtype H3N2 was inhibited by G4-SA at a 51–170 fold lower concentration than monomeric SA and sialoside (table 3). Hemagglutination caused by influenza A viruses expressing a H2N2 subtype was not inhibited at any concentration of G4-SA. Hemagglutination by some viruses of the H1N1 subtype was inhibited at concentrations 32–43 fold less than that for SA controls. Of interest, 2 viruses of the H1N1 subtype, PR 8/34 and FM/1/47, produced hemagglutination that was not inhibited by G4-SA. Modifying the G4-SA by using long PEG linker chains produced polymer decoys that were ineffective at inhibiting any of the influenza subtypes at concentrations at or below that of monomeric SA.

The positive control for the hemagglutination-inhibition reactions using SA and sialoside gave consistent inhibition at a concentration of 10 m*M,* regardless of virus serotype.

Toxicity testing in vivo. All the mice tolerated the G4-SA conjugates at all concentrations without demonstrating toxicity, compared with the PBS control group. Gross and microscopic pathologic examination of the lungs revealed that they remained normal in appearance after challenge (figure 3).

Assessment of dendrimer SA conjugates in vivo. G4-SA was effective in preventing influenza A X-31 infection in the murine influenza A virus pneumonitis model. Seventeen mice were exposed to a suspension of virus alone, whereas another 17 mice were exposed to a suspension of a lethal dose of viruses mixed with G4-SA (test). The 14-day postexposure survival rate was 100% in the test group, whereas it was only 6% in the control group. The deaths in the control group occurred within 7 days.

The second experiment involved 2 mouse-adapted doses of X-31. Mouse-adapted influenza A X-31 virus (suspended in PBS) was mixed with an equal volume of G4-SA to a final G4-SA concentration of 9 μg/g of mouse body weight, whereas control mice received doses of virus only. The control mice did not survive. The surviving mice in the virus and G4-SA group showed no significant signs of illness (figure 4), and the lungs of the test mice did not display abnormalities. Lungs taken from the terminally moribund control mice showed >75% consolidation (figure 5). The results of these experiments are presented in table 1.

When G4-SA was tested with mouse-adapted influenza A/AA/6/60 virus in the same model system and with same experimental design, there was no significant inhibition of infection. These experiments demonstrate the inability of dendrimer conjugated with SA to inhibit viral infection by this H2N2 virus strain. These experiments are also summarized in table 1.

It was also found that G4-SA was effective in preventing influenza A virus infection in a dose-dependent fashion. The number of surviving mice increased with the amount of G4-SA administered, with survival reaching a threshold at a concentration of 1.8 μg/mg of mouse body weight and a calculated 50% effective dose of 0.96 μg of G4-SA per mouse (table 2).

All G4-SA–pretreated mice tested with non–mouse-adapted X-31 virus survived the 14 day trial, whereas control animals that were administered PBS as a pretreatment before virus did not. However, G4-SA–pretreated mice challenged with the mouse-adapted strain of X-31 virus were not protected and did not survive the 14-day trial. As expected in this latter experiment, control mice pretreated with PBS and challenged with the mouse-adapted virus also did not survive.

Computational modeling and simulations. Three-dimensional structures of the hemagglutinin proteins and the final configuration of the G4-SA are shown in (figure 6). Of the hemagglutinin protein chains examined, only the amino acid sequences of the HA1 protein A1/FM/1/47 and AA 6/60 were available. The primary receptor sites (figure 6, *arrows*) are relatively distant from the hemagglutinin HA2 chain, and, not surprisingly, HA2 was not found to affect the intermolecular energy calculations. The conformation of the primary binding sites of hemagglutinin subtype H1N1 are very similar to each other and appear to be closer to subtype H3N2 than H2N2. The final configuration of G4-SA after simulation found that most SAs are located on the outside of dendrimer platform. One of the most peripheral SAs on the G4-SA model was chosen for the interaction energy calculations.

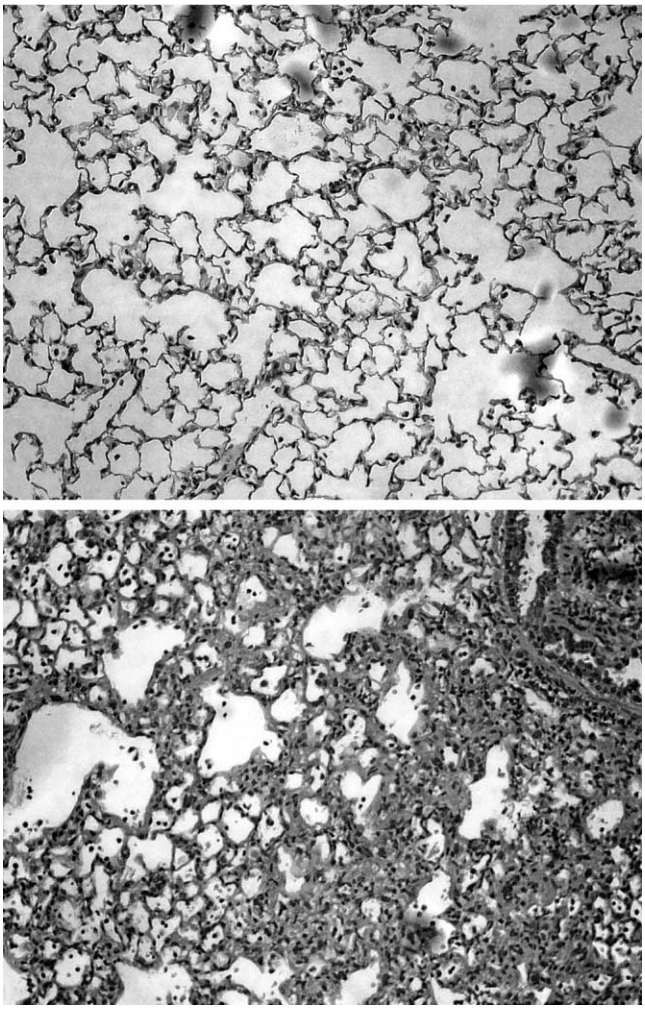

Figure 5. *A,* Mouse lung tissue section 14 days after application of generation 4 sialic acid–conjugated polyamidoamine dendrimer and influenza A X-31 (original magnification, ×200). *B,* Mouse lung tissue 7 days after application of X-31 virus control (original magnification, ×200).

Figure 7 shows the interaction energy range between G4-SA and hemagglutinin subtype H3N2 (strain A/Aichi/2/68). Other influenza A hemagglutinin strains were similarly evaluated for interaction energy calculations. We compared the interacting residues of each hemagglutinin to confirm the consistency of the interaction, and there was no significant difference in the

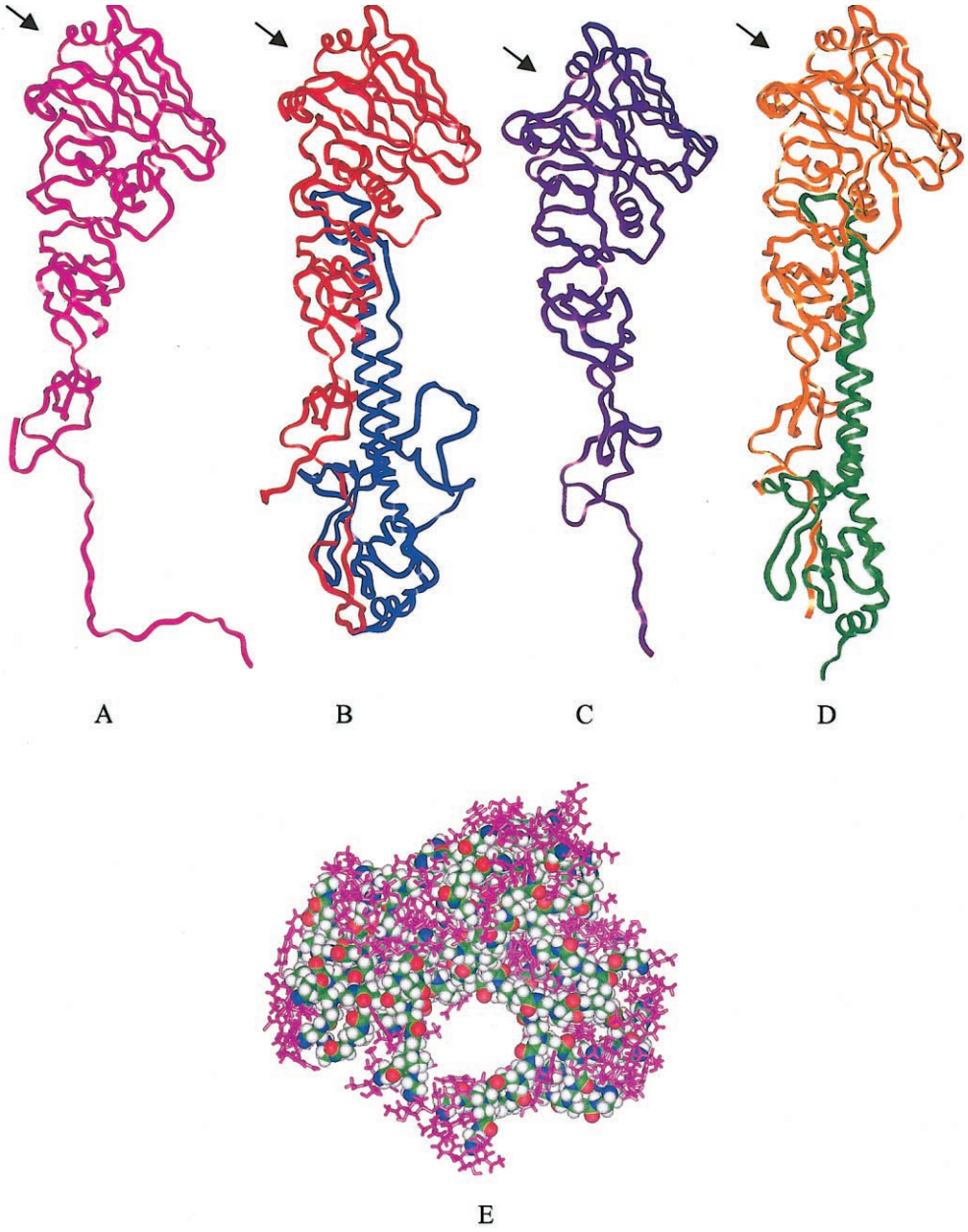

Figure 6. Hemagglutinin models of subtype H1N1 A1/FM/1/47 *(A)* and A/WS/33 *(B),* subtype H2N2 AA 6/60 *(C),* and subtype H3N2 A/Aichi/2/68 (*D;* a structure from the Protein Data Bank, available at http://www.rcsb.org/pdb/). *E,* Generation 4 sialic acid–conjugated polyamidoamine dendrimer (G4-SA) after a 100-picosecond molecular dynamics simulation (pink color indicates sialic acid). *Arrows,* primary binding sites.

number of atoms involved. The calculated energies of different strains are presented in table 4. Hemagglutinin subtypes H2N2 and H3N2 present the highest and lowest interfacial energy values, respectively, whereas the interfacial energies of subtype H1N1 are in between these values. Although strains A1/FM/1/47 and A/WS/33 are of the same subtype, their interfacial energies were found to be dramatically different and correlated with their respective abilities to inhibit virus binding.

Discussion

Small-molecule inhibitors have become important therapeutic agents for treating influenza A. For example, neuraminidase inhibitors have been shown to be effective in human trials since the middle of the 1990s [2]. Molecular decoys have been theorized to be an effective means of inhibiting viral adhesion and infection of cells. The most effective use of this technology

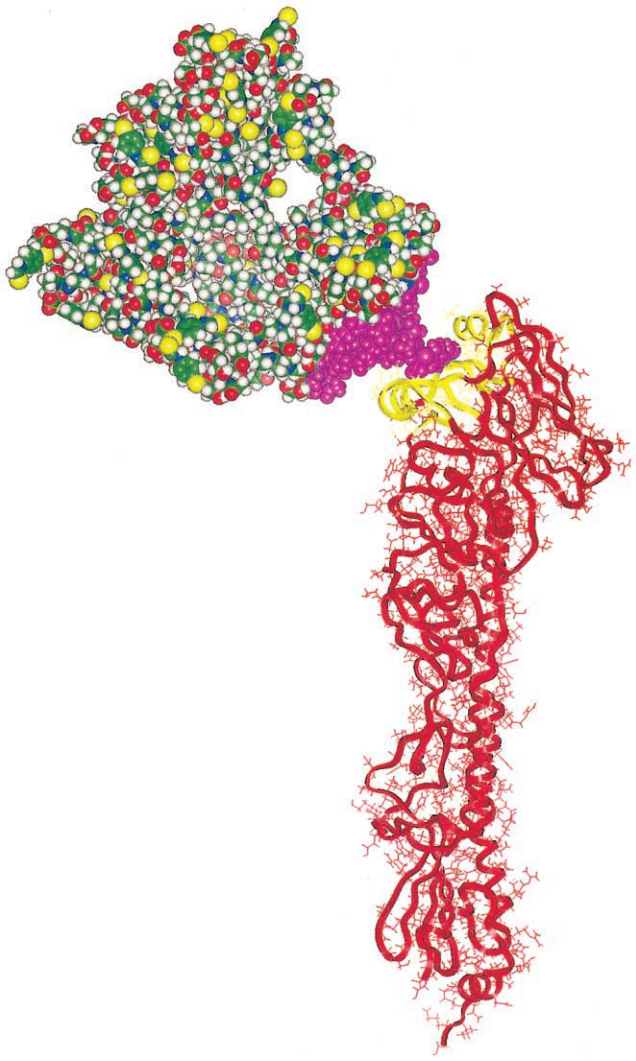

Figure 7. The interaction between hemagglutinin strain A/Aichi/2/68 and G4-SA. The pink in the dendrimer conjugate and yellow in hemagglutinin are the intermolecular energy calculation ranges.

would, theoretically, occur with viruses that share a common receptor target, such as influenza viruses, where a single decoy molecule could theoretically inhibit all strains of the virus.

Although it has been established that dendrimers with SA conjugates have the ability to inhibit red blood cell agglutination by influenza virus in the HIA [4], the ability of these agents to prevent influenza infection from a wide array of virus strains in vivo had not been assessed. The evaluation of virus binding in vitro yielded interesting data (table 3), because 3 influenza virus strains of subtype H3N2 are effectively inhibited by G4-SA. In contrast, 3 influenza virus strains of subtype H2N2 showed no binding inhibition, whereas the response from H1N1 subtypes of influenza was mixed; some were completely inhibited, whereas others showed no inhibition even at high concentrations of polymeric SA. This suggests a unique structural specificity for the binding between hemagglutinin and SA conjugated to the surface of the dendrimer. Given the structural differences among the various hemagglutinin subtypes, the variation in response to the inhibitor is not entirely surprising. However, the strain-specific variations in hemagglutination inhibition within the H1N1 subtype are more remarkable. One must conclude that the few amino acid differences among these H1 proteins generate enough variation, in binding cleft orientation or other structural alternation, to totally block binding of the G4-SA to H1.

The ability of sialated dendrimers to have polyvalent binding to virus hemagglutinin appears to create a high affinity interaction that could effectively compete with host cells and cause inhibition of viral adhesion. In addition, it has been theorized that dendrimers bound on the surface of the virus may cause nonspecific steric inhibition of the virus binding to cells [4]. Although these are 2 potential reasons why G4-SA succeeds at inhibiting influenza infection, understanding the subtler puzzle of how G4-SA discriminates between and within different virus subtypes is crucial to the use of this agent as a therapeutic. As with a vaccine, if the decoy does not consistently inhibit all serotypes of an infecting virus, one would need to predict potential exposures and design decoys to use for each therapeutic application. In addition, understanding this phenomenon could direct modification of the polymeric inhibitor to increase the breadth of its inhibitory capability. Recent molecular modeling data and interfacial energy calculations point to reasons for this discrimination. The interfacial energy calculations (table 4) show that the inhibition ability of sialated dendrimer is better when the interfacial energy between the SA and the hemagglutinin binding cleft is more stable (i.e., lower); inhibition did not occur with strains where higher interfacial energy suggested instability. Hemagglutinin activity in vivo depends on the formation of a trimer, and the interfacial energy differences we calculated for the monomer subunit should be increased further by trimerization. Since these interfacial energy differences were calculated from only one configuration of the sialated dendrimer acting on the primary binding site of hemagglutinin, more studies need to be done. However, these initial studies suggest that molecular modeling may be one means to evaluate the

Table 4. Interfacial energies between sialated dendrimer and hemagglutinin from computer models with the minimum sialic acid concentration of sialated dendrimer for viral inhibition obtained from previous experiments.

Virus subtype, strain	Interfacial energy, kcal	Minimum sialic acid concentration, m*M*
H1N1		
A1/FM/1/47	1.25	NI
A/WS/33	−1.66	0.313
H2N2		
AA 6/60	1.30	NI
A/Aichi/2/68	−2.86	0.117

NOTE. NI, not inhibited (i.e., ⩽2.5 m*M*).

interaction differences between G4-SA and the individual influenza strains. The failure of the sialoside on PEG linkers to aid in inhibition reinforces the complexity of this issue.

Pretreatment of the animals' nares with the decoy molecule prevented infection from non–mouse-adapted X-31 virus, which suggests that this material might be useful for prophylaxis. However, the studies investigating the pretreatment of mice with G4-SA show that with the virulent, mouse-adapted X-31 virus, prophylaxis was not successful. This may be the result of the mouse-adapted X-31 virus' extremely low lethal dose, where even a few virions can cause productive infection. Future experiments are necessary to fully characterize the efficiency of pretreatment with G4-SA as a prophylactic means of preventing infection with several strains of influenza A virus.

A concern with these studies was that the SA-G4 dendrimers might induce an immune response that provided protection in mice. We do not believe this to be the case, because studies in our laboratory found no immune response to dendrimer in mice. In addition, prior studies examining immunization of mice with dendrimer in complete Freund's adjuvant, both alone and in conjunction with bovine serum albumin have not been able to elicit an antibody response in vivo (J. Kukowska-Latallo, personal communication). This is likely due to the absence of a T cell epitope in the dendrimer. Thus, an immune response to the dendrimer is not likely to be an issue in these studies.

The experiments reported in this paper represent the first documentation of the function of dendrimer conjugates as antiinfective agents in vivo. These in vivo studies demonstrate the ability of G4-SA to protect against experimental infection by influenza A X-31 H3N2 virus in mice. This was fully anticipated by in vitro experimentation, as was the inability of G4-SA to prevent infection against influenza A/AA/6/60 (H2N2) virus. This suggests that inhibiting SA binding by hemagglutinin is adequate to prevent infection by influenza virus. In addition, it appears that in vitro hemagglutination-inhibition data reliably predicts physiologic binding events in vivo. These findings should aid in the further designs of viral inhibitors.

In summary, our study shows that polyvalent SA conjugated dendrimers can prevent pulmonary infection with influenza A virus. Of importance, we found that subtype specificity and strain variance played a critical role in determining the ability of G4-SA to inhibit hemagglutination, and these findings have consequences for the development of these types of inhibitors as therapeutic agents. Molecular modeling of the hemagglutinin/decoy interaction provides information that may help to explain the experimental observations, and could be useful in evaluating these phenomena.

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